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Fermentation of Cellulose and Cellobiose by *Clostridium thermocellum* in the Absence and Presence of *Methanobacterium thermoautotrophicum*

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The fermentation of cellulose and cellobiose by *Clostridium thermocellum* monocultures and *C. thermocellum/Methanobacterium thermoautotrophicum* cocultures was studied. All cultures were grown under anaerobic conditions in batch culture at 60°C. When grown on cellulose, the coculture exhibited a shorter lag before initiation of growth and cellulolysis than did the monoculture. Cellulase activity appeared earlier in the coculture than in the monoculture; however, after growth had ceased, cellulase activity was greater in the monoculture. Monocultures produced primarily ethanol, acetic acid, H_2, and CO_2. Cocultures produced more H_2 and acetic acid and less ethanol than did the monoculture. In the coculture, conversion of H_2 to methane was usually complete, and most of the methane produced was derived from CO_2 reduction rather than from acetate conversion. Agents of fermentation stoppage were found to be low pH and high concentrations of ethanol in the monoculture and low pH in the coculture. Fermentation of cellobiose was more rapid that that of cellulose. In cellobiose medium, the methanogen caused only slight changes in the fermentation balance of the *Clostridium*, and free H_2 was produced.

Cellulose is the most abundant of the earth's biopolymers and is a major component of industrial and municipal waste. Recent interest in the use of anaerobic digestion for the conversion of cellulosic wastes to methane has prompted investigation of the characteristics of bacterial cellulose fermentation.

Methanogenic bacteria have been shown to alter the metabolic patterns of chemoorganotrophic bacteria both in the rumen (11, 22) and in mixed cultures of defined bacterial composition (3, 6, 19, 20, 23). It has been suggested that methanogens act as "electron sinks" by making it energetically feasible for the chemoorganotroph to dispose of electrons as H_2 rather than as other reduced products, such as ethanol (23). Thus, in anaerobic ecosystems, H_2 produced by chemoorganotrophs is oxidized to methane by the methanogens. This type of microbial interaction has been called interspecies hydrogen transfer (12, 23). Studies on the interaction between chemoorganotrophic and methanogenic bacteria have been confined to mesophilic systems grown on soluble substrates such as glucose (20, 23) or ethanol (19). We report here on the interaction in batch culture between two thermophiles, *Clostridium thermocellum* and *Methanobacterium thermoautotrophicum*, when grown on cellulose or cellobiose.

C. thermocellum LQ8 ferments cellulose and cellodextrins (but not glucose or xylose) to produce primarily H_2, CO_2, ethanol, and acetic acid (T. K. Ng and J. G. Zeikus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K123, p. 157). *M. thermoautotrophicum* is a chemolithotrophic autotroph which requires H_2 as an electron donor in methanogenesis. Carbon dioxide is required for growth and is the preferred electron acceptor in methanogenesis (25, 26).

MATERIALS AND METHODS

Organisms. *C. thermocellum* LQ8 was generously provided by L. Y. Quinn, Department of Bacteriology, Iowa State University, Ames, Ia. *M. thermoautotrophicum* ΔH is the type strain described by Zeikus and Wolfe (26).

Anaerobic methods. The anaerobic culture technique of Hungate (10) as modified by Bryant (2) was used throughout the course of this work. Anaerobic culture tubes (18 by 142 mm, Bellco) contained 10 ml of medium. Flask cultures were grown in 250 Erlenmeyer flasks that contained 100 ml of medium. All vessels were sealed with neoprene stoppers (Sargent-Welch).

Growth of organisms. *C. thermocellum*, with or without *M. thermoautotrophicum*, was grown in CM3 medium. The composition of CM3 was (per liter): cellulose (Machery and Nagel 300, thin-layer chromatography grade), 9.72 g; yeast extract, 2.0 g; $(NH_4)_2SO_4$, 1.3 g; KH_2PO_4, 1.5 g; $K_2HPO_4 \cdot 3H_2O$, 2.9

g; $MgCl_2 \cdot 6H_2O$, 1.0 g; $CaCl_2$, 0.15 g; 0.2% resazurin (Eastman Kodak), 1.0 ml; 5% $FeSO_4$, 0.025 ml. The pH was adjusted to 7.8 with NaOH prior to dispensing 9.6 ml of medium into test tubes under constant, vigorous gassing with N_2. A 1.25% cysteine hydrochloride/1.25% $Na_2S \cdot 9H_2O$ solution (0.4 ml) was then added to each tube, and the tubes were secured in a press before autoclaving for 15 min at 15 lb/in^2. Larger volumes of CM3 were autoclaved before reduction in cotton-plugged Erlenmeyer flasks. After sterilization, flasks were gassed under N_2, stoppered, and reduced. Cellobiose medium was prepared by replacing cellulose with filter-sterilized solutions of cellobiose.

Stock cultures of *C. thermocellum* were maintained through biweekly transfer of 1 ml of culture into fresh CM3. Since this strain did not utilize glucose, contamination checks were performed in CM3 that contained glucose rather than cellulose. Turbidity and gas production after 72 h were taken as evidence of contamination.

Stock cultures of *M. thermoautotrophicum* were grown in 500-ml anaerobic shake flasks (8) that contained 200 ml of LPBM III. The composition of LPBM III was (per liter): KH_2PO_4, 0.15 g; $Na_2HPO_4 \cdot 7H_2O$, 1.05 g; NH_4Cl, 0.53 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; 0.2% resazurin, 1 ml; mineral elixer B, 10 ml; $Na_2S \cdot 9H_2O$, 0.5 g. Mineral elixer B contained the following (per liter): nitrilotriacetic acid, 1.5 g; $FeCl_2$, $4H_2O$, 0.3 g; $MnCl_2 \cdot 4H_2O$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 0.17 g; $ZnCl_2$, 0.1 g; $CuCl_2$, 0.02 g; H_3BO_3, 0.01 g; $NaMoO_4$, 0.01 g. Flasks were shaken at 20 rev/min in a 60°C gyratory water bath, while being continually gassed at approximately 20 cm^3/min with a mixture of 80% water/20% CO_2 (vol/vol). Transfers were made at 2- to 5-day intervals.

Experimental cultures were prepared by inoculating approximately 10^6 *C. thermocellum* cells (total count, equivalent to 0.2 to 0.5 ml from an exponential-phase culture) into 10 ml of reduced CM3. Cocultures were usually prepared by simultaneous inoculation of 10^6 cells (total count) of each species. Volume differences between monocultures and cocultures were corrected via addition of sterile LPBM III. Cultures were flushed with N_2 for 30 min prior to inoculation into fresh media. All cultures were incubated at 60°C in an upright position without shaking.

Growth was measured as optical density (OD) at 525 nm in a Bausch and Lomb Spectronic 20 colorimeter. Cultures that contained cellulose were blended in a Vortex mixer and allowed to stand upright for 3 h prior to OD measurement. Cultures that contained cellobiose were measured immediately after blending in a Vortex mixer. Cell counts were made with a Petroff-Hauser counter.

Product analysis. Experimental culture tubes were analyzed for H_2, CH_4, CO_2, $^{14}CH_4$, and $^{14}CO_2$ by the gas chromatography-gas proportional counting method of Nelson and Zeikus (17). Culture gas phase (0.4 ml) was injected directly into the gas chromatograph, using a 1-cm^3 glass hypodermic syringe and a pressure-lock fitting (Supelco). All gas quantities were adjusted to take into account their theoretical solubilities and, in the case of CO_2, the effect of the bicarbonate equilibrium at the appropriate pH.

Alcohols and volatile fatty acids were measured as follows. Samples (0.15 ml) were removed from the culture vessels and placed in cooled, 0.4-ml, capped plastic centrifuge tubes (Arthur Thomas). After centrifugation at 20,000 × *g* for 1 min, 0.15 ml of 1.0 N H_3PO_4 was added to the supernatant and 2 μl of the mixture was injected into a Packard 419 gas chromatograph. The instrument was equipped with a flame ionization detector and a Teflon column (1.8 m by 2 mm [inner diameter]) containing 3% Carbowax 20M/0.5% H_3PO_4 on 60/80 Carbopack B (Supelco). The following parameters were selected for the analysis: column temperature, 170°C; injector temperature, 200°C; carrier gas, 60 cm^3 of helium per min; H_2 flow rate, 35 cm^3/min; air flow rate, 300 cm^3/min. Quantitative analysis was achieved by comparison of sample peak heights to those of standards prepared in 0.5 N H_3PO_4.

Lactic acid was measured by the colorimetric method of Barker and Summerson (1). Qualitative detection of formic acid (0.5 mM sensitivity) was attempted using thermal conductivity gas chromatography and a column that contained 15% SP-1220/1% H_3PO_4 on acid-washed Chromosorb W, as described by Hauser and Zabransky (9).

[^{14}C]acetic acid in culture supernatants was separated by elution through a column (15 cm by 4 mm) of Dowex AG1-X10, formate form (BioRad), using an increasing formic acid gradient as described by LaNoue et al. (14). Fractions of 2.5 ml were collected, 1.0 ml of which was added to 9 ml of Aquasol (New England Nuclear) and counted in a Packard 3375 liquid scintillation counter. Counting efficiency was 82%, as determined with a [^{14}C]toluene standard (New England Nuclear).

Residual cellulose was measured as follows. Cultures were centrifuged at 25,000 × *g* for 15 min, and the supernatant was drawn off gently with a Pasteur pipette. The pellet was resuspended in 8% formic acid to lyse the cells. This solution was then passed through preweighed 0.45-μm membrane filters (Millipore Corp.). The filters were dried at 60°C to constant weight, and the residual cellulose was determined by difference.

Reducing sugars were determined by the method of Miller et al. (16). The assay mixture contained 1.0 ml of citrate buffer (0.10 M, pH 5.0), 0.20 ml of culture supernatant, and 3.0 ml of dinitrosalicylic acid reagent (DNS). Glucose solutions were used as standards.

Analysis of cellulolytic activity. Because cellulase has been shown to bind to cellulose (10, 15), cellulolytic activity was measured in two ways. (i) In the supernatant assay, cultures were centrifuged at 20,000 × *g* for 1 min, and 0.20 ml of the supernatant was added to 1.0 ml of 2% sodium carboxymethylcellulose (Sigma) in citrate buffer (0.10 M, pH 5.0). After incubation at 60°C for 30 min, the amount of reducing sugars liberated was determined as above. (ii) In the culture assay, culture samples (1 ml) were withdrawn from the fermentation vessel and added to vials that contained 1.0 ml of phosphate buffer (0.05 M, pH 6.0) and 3.0 × 10^5 dpm of [U-^{14}C]cellulose (ICN, 2.4 mCi/mmol). The cellulose was added as a sterile suspension, prepared by gentle grinding with water in a glass tissue homoge-

nizer followed by autoclaving at 15 lb/in² for 15 min. Reaction vials were incubated at 60°C in a reciprocating shaker operating at 40 strokes/min. After 2 h of incubation, 1.0 ml of the reaction mixture was removed and passed through a 0.45-μm membrane filter. The filter was washed with 1.0 ml of water. Filters were counted in 9 ml of Aquasol + 1 ml of water. Samples (0.5 ml) of filtrate were counted in 9 ml of Aquasol + 0.5 ml of water. The percentage of ^{14}C solubilized was then adjusted to compensate for the varying amount of residual unlabeled cellulose added from the whole culture to the reaction vial. Residual cellulose was determined as described above. Results were expressed as milligrams of cellulose solubilized per milliliter of culture per hour.

RESULTS

Visual characteristics of the cellulose fermentation. A lag in initiation of cellulolytic activity was observed upon inoculation of *C. thermocellum* into fresh media. The length of this lag period varied from 4 to 48 h, depending on the growth phase of the organism at the time of transfer. Initial evidence of fermentation was a "fluffing" of the cellulose and the release of discrete gas bubbles. A yellow pigment which bound tightly to the cellulose was then produced. As the fermentation became more vigorous, the cellulose was gradually solubilized and the overlying liquid became turbid due to cell growth. Cessation of growth and fermentation usually occurred within 3 days of its inception. During the course of fermentation, gas pressures sufficient to explode the culture flasks were often produced. Coculturing of *C. thermocellum* with *M. thermoautotrophicum* resulted in a shortening of the lag period and in a more vigorous fermentation during the first 3 days of incubation.

Products of cellulose fermentation. Figure 1 shows the time course of cellulose fermentation by cultures of *C. thermocellum* in the absence and presence of *M. thermoautotrophicum*. Relative to the *C. thermocellum* monoculture, the *C. thermocellum/M. thermoautotrophicum* coculture exhibited a shorter lag before the initation of cellulolysis. However, the total amount of cellulose solubilized after 100 h of incubation was greater in the monoculture than in the coculture. At the concentration of cellulose employed (0.82%), neither culture degraded all of the substrate. Though both cultures reached similar final optical densities, the coculture exhibited a shorter lag and more rapid growth in the early stages of the fermentation. The cellulose fermentation was accompanied by a large decrease in culture pH. Relative to the monoculture, the coculture showed an earlier pH drop and reached a lower final pH.

Figure 2 illustrates the rate of product formation during cellulose fermentation. The main products in the monoculture—ethanol, acetic acid, H_2, and CO_2—were produced at relatively constant rates for about 20 h after the onset of fermentation. Lactic acid was an early product, whereas butyric acid was produced after formation of most other products had ceased. Product formation in the coculture differed dramatically from that of the monoculture. As in the monoculture, acetic acid, lactic acid, and ethanol were formed early in the fermentation and butyric acid appeared as a late product. However, acetic acid production increased threefold, whereas ethanol production decreased fivefold. No free H_2 was detected in the coculture. If one assumes that one equivalent of methane is formed by the oxidation of four equivalents of H_2, approximately twice as much H_2 was produced in coculture as in the monoculture. Both the monoculture and coculture produced trace quantities of butanol and succinic acid. Neither culture contained detectable levels of formic acid or free reducing sugars.

The fermentation balance of cultures incubated for 100 h is shown in Table 1. Although good statistical agreement (standard deviations of less than 10%) was obtained for replicates within a given experiment, fermentation balances were found to vary somewhat from experiment to experiment. In some cases, cocultures produced free H_2, formed less methane and acetic acid, and formed more ethanol than did the coculture shown above. These characteristics were observed under any of the following conditions: (i) initial ratio of *C. thermocellum* cell number/*M. thermoautotrophicum* cell number exceeded 5; (ii) a stationary-phase culture of *M. thermoautotrophicum* was inoculated simultaneously with an exponentially growing culture of *C. thermocellum*; (iii) *M. thermoautotrophicum* was inoculated into a *C. thermocellum* monoculture after fermentation had already begun and the pH had dropped below 6.8.

Cellulolytic activity during fermentation. Figure 3 illustrates cellulolytic activity of the monoculture and coculture under two different assay conditions. During the first 50 h of incubation, both solubilization of [^{14}C]cellulose by whole cultures and the production of DNS-detectable reducing sugars from CMC by culture supernatants was significantly greater in the coculture than in the monoculture. Both cultures exhibited similar net activity maxima (5 to 6 mg of cellulose solubilized per ml of culture per h), but activity declined earlier in the coculture than in the monoculture. Uninoculated controls contained some soluble

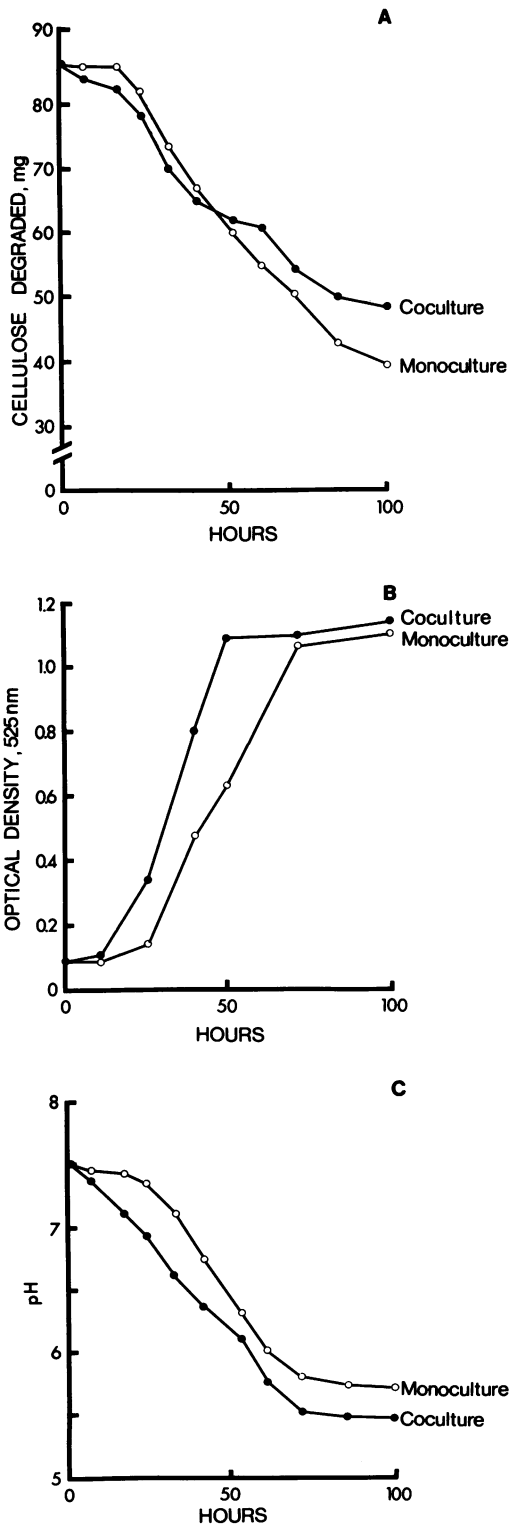

FIG. 1. *Fermentation of cellulose by C. thermocellum monocultures and C. thermocellum/M. thermoautotrophicum cocultures. Cultures were grown in anaerobic tubes that contained 12 ml of medium. (A) Residual cellulose; (B) optical density at 525 nm; (C) culture pH.*

[^{14}C]cellodextrins which were present in the [^{14}C]cellulose added to the initial assay mixture.

Conversion of CO_2 and acetate to methane. CO_2 and acetate are considered to be the major carbon precursors of methane in natural ecosystems and in pure cultures of some methanogenic bacteria (5, 13, 25). Conversion of these substrates to methane by *M. thermoautotrophicum* in coculture was investigated by adding $Na_2{}^{14}CO_3$ or [U-^{14}C]sodium acetate to tubes inoculated with the mixed culture. At 0,

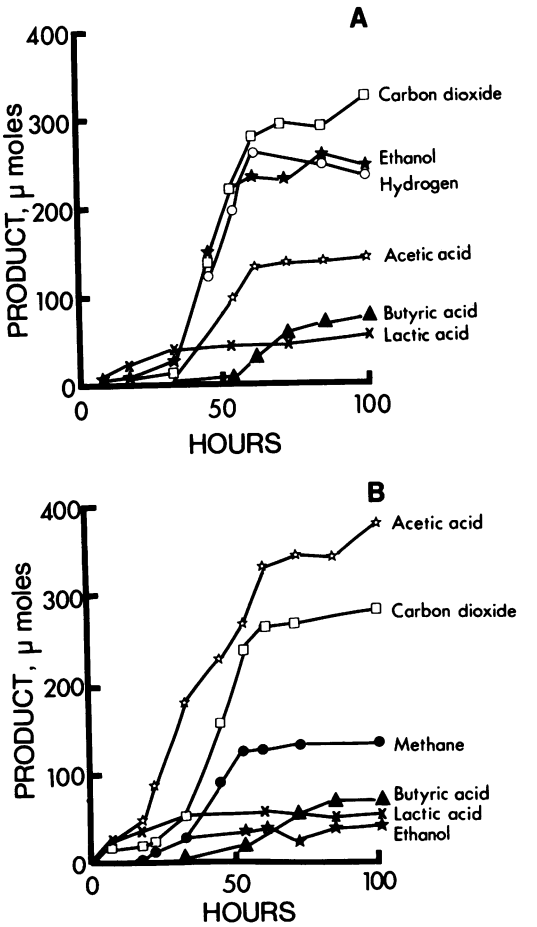

FIG. 2. *Products of cellulose fermentation by C. thermocellum grown in the absence (A) and presence (B) of M. thermoautotrophicum. Cultures were grown in anaerobic tubes that contained 12 ml of medium. Results are expressed in micromoles of product per tube.*

TABLE 1. *Products of cellulose fermentation by C. thermocellum in the absence and presence of M. thermoautotrophicum*

Product	Amt produced[a]	
	C. thermocellum	*C. thermocellum* + *M. thermoautotrophicum*
H_2	85.2	0
Methane	0	56.2
Ethanol	88.1	18.0
Acetic acid	47.9	153.0
Butyric acid	25.2	24.0
Lactic acid	12.9	14.1
CO_2	113.2	111.1
Cellulose degraded (mg)[b]	45.0	38.9
O/R index[c]	0.73	1.13
C_2/C_1	1.20	1.02
C recovery	0.88	1.08
H recovery	0.97	1.02

[a] Results are mean values of duplicate tubes after 100 h of incubation, and are expressed as millimoles per 100 mmol of "anhydroglucose equivalents" (the monomeric form of cellulose, molecular weight 162 g/mol) fermented. Butanol, isopropanol, and succinic acid were trace products (<1.3 mmol/100 mmol of anhydroglucose) and were not tabulated. Formic acid was not detected (sensitivity ~3 mmol/100 mmol of anhydroglucose).

[b] Initial quantity of cellulose was 93 mg.

[c] Calculated by the method of Wood (24).

12, and 36 h, the cultures were analyzed for unlabeled and ^{14}C-labeled CO_2, acetate, and methane. Results are presented in Table 2. Because CO_2, acetic acid, and methane were continually produced during the fermentation, their specific activities all decreased during the course of the experiment. In tubes that contained added $Na_2{}^{14}CO_3$, the specific activity of the methane resembled that of the CO_2. In tubes that contained added [^{14}C]acetate, little $^{14}CH_4$ was evolved, and the specific activity of the methane was less than 1% of that of the acetate.

Effect of fermentation products on cellulose fermentation. Since *C. thermocellum* produced large quantities of H_2, it was of interest to determine if H_2 was inhibitory to the organism. Cultures were grown in CM3 under N_2, H_2, Ar, or CO_2 atmospheres. The results are shown in Table 3. Under these gas phases, no significant differences were observed among any of a number of fermentation characteristics. H_2 did not inhibit growth or fermentation. Other experiments indicated that addition of H_2 to cultures actively growing under N_2 did not alter the rate of growth or cellulose degradation.

Ethanol exerted an inhibitory effect on fermentation by both culture types. Flasks of CM3 that contained 40 mM ethanol were inoculated with *C. thermocellum*. No visible growth or cellulolysis occurred after 72 h. Addition of ethanol (final added concentration 44 mM) to active *C. thermocellum* monocultures caused a cessation in gas production. Similar addition to the coculture resulted in a 34% decrease in gas production. Cellulose fermentation in both monoculture and coculture was inhibited by low pH. Fermentation was not initiated in media which had an initial pH below 6.5. Adjusting the pH of active cellulose fermentation to 5.0 (by the addition of HCl) caused a stoppage in gas production.

Fermentation of cellobiose. The fermentation of cellobiose by *C. thermocellum* in the absence and presence of *M. thermoautotrophicum* differed markedly from the fermentation of cellulose. Cellobiose fermentation was not accompanied by production of a yellow pigment or discrete gas bubbles, although large gas pressures were produced. The time course of cellobiose fermentation was much more rapid than was that of cellulose: minimum doubling times of *C. thermocellum* were 2.1 h on cellobiose and 11 h on cellulose. As indicated in Fig. 4, both the monoculture and coculture exhibited nearly identical rates of growth and of pH decrease when grown on cellobiose.

Figure 5 shows the rate of product formation during cellobiose fermentation by the monoculture and coculture. The monoculture produced more ethanol than did the coculture, although both cultures formed similar amounts of CO_2, acetic acid, and butyric acid. Small amounts of methane were produced in the coculture, but after 15 h methanogenesis ceased and the production of H_2 and CO_2 rapidly increased.

DISCUSSION

In both the *C. thermocellum* monoculture and the *C. thermocellum/M. thermoautotrophicum* coculture, the fermentation of cellulose was much slower than that of cellobiose. Reducing sugars were not detected duing cellulose fermentation in either monoculture or coculture. These data show that the rate-limiting step in the fermentation of cellulose by *C. thermocellum* is the solubilization of cellulose. Free H_2 was not usually detected in the coculture grown on cellulose, indicating that methanogenesis was limited by the rate of H_2 production by *C. thermocellum*. However, the increased growth rate of *C. thermocellum* on cellobiose was sufficient to cause a rapid buildup of H_2, even in coculture with *M. thermoautotrophicum*. Methanogenesis in the cellobiose cocul-

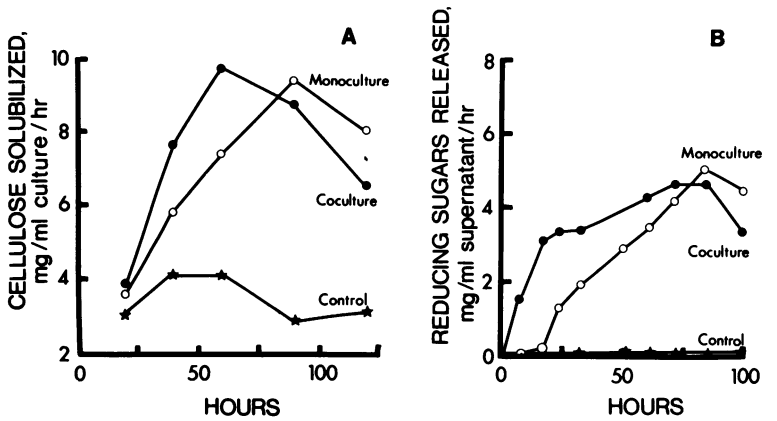

FIG. 3. *Cellulolytic activity of C. thermocellum monocultures and C. thermocellum/M. thermoautotrophicum cocultures. Cultures were grown in flasks and samples were withdrawn periodically and assayed as described in Materials and Methods. (A) Whole culture assay; (B) supernatant assay.*

TABLE 2. *Conversion of CO_2 and acetate to methane by C. thermocellum/M. thermoautotrophicum cocultures*[a]

Determination	$Na_2{}^{14}CO_3$ added			[^{14}C]sodium acetate added		
	0 h	12 h	36 h	0 h	12 h	36 h
CH_4 (μmol)[b]	0	9.1	114.1	0	8.6	77.7
CO_2 (μmol)[b]	1.4	5.0	147.2	1.8	5.7	170.7
Acetic acid (μmol)[c]	17.9	46.5	169.8	25.2	51.0	186.8
$^{14}CH_4$ (cpm)[b]	0	29,190	58,900	0	2,015	2,430
$^{14}CO_2$ (cpm)[b]	11,570	21,620	52,860	0	1,960	3,000
[^{14}C]acetic acid (cpm)[c]	0	0	0	1.843×10^6	1.430×10^6	1.321×10^6
Sp act CH_4 (cpm/μmol)		3,210	520		230	30
Sp act CO_2 (cpm/μmol)	8,260	4,360	360		340	20
Sp act acetic acid (cpm/μmol)				73,130	28,040	7,070

[a] Tubes contained 10 ml of reduced CM3 and 1.85×10^6 cpm of $Na_2{}^{14}CO_3$ or [U-^{14}C]sodium acetate, and were inoculated with 3.0×10^6 *C. thermocellum* cells and 1.2×10^6 *M. thermoautotrophicum* cells. Results are mean values of triplicate tubes. Initial gas phase N_2.

[b] Gas phase only.

[c] Culture supernatant only.

TABLE 3. *Effect of initial gas phase on the fermentation of cellulose by C. thermocellum*[a]

Initial gas phase	CO_2 produced[b]	Final OD_{525}	Final culture pH	Cellulose solubilized (mg)
H_2	180.8 ± 3.8	1.07 ± 0.12	5.91	40.9 ± 5.6
N_2	167.1 ± 24.1	1.08 ± 0.09	5.95	38.3 ± 1.2
Ar	191.5 ± 12.9	1.21 ± 0.15	5.69	44.2 ± 5.6
CO_2	ND[c]	1.18 ± 0.15	5.77	35.5 ± 4.7

[a] Cultures were analyzed after 108 h of incubation. Initial conditions: OD_{525} = 0.26 to 0.28, pH = 7.75, total cellulose = 83.3 mg. Results are mean values from triplicate tubes, plus or minus one standard deviation.

[b] Expressed in micromoles in total gas phase.

[c] ND, Not determined.

ture was apparently limited by the growth rate of the methanogenic bacteria.

C. thermocellum ferments cellulose or cellobiose primarily to H_2, CO_2, ethanol, and acetic acid. We suggest that coculturing of the *Clostridium* with *M. thermoautotrophicum* on cellulose causes a shift in the conversion of acetyl-CoA from ethanol to acetic acid. This in turn

would result in more electrons being available for production of H_2, and ultimately, methane. Most of the methane was produced by reduction of CO_2; virtually no methanogenesis from acetate was observed. Cellulose-grown cocultures produced greater quantities of acetic acid and lowered the pH more than did the monocultures. Both monoculture and coculture produced similar quantities of lactic and butyric acids. Butyric acid is produced late in the fermentation as a biochemical alternative to production of ethanol or acetic acid. Theoretically, the conversion of two equivalents of acetyl CoA to butyric acid allows the organism to oxidize two reduced nicotinamide adenine dinucleotides (NADHs) and produce one adenosine 5'-triphosphate (ATP) while producing only one equivalent of acid (24). Low pH was probably responsible for the earlier cessation of fermentation in the coculture; fermentation in the monoculture apparently ceased due to both low pH and high concentration of ethanol.

We did not observe a significant metabolic interaction between the *Clostridium* and the methanogen during growth on cellobiose. This finding may be of general ecological significance. In nature the decomposition of organic matter might be limited by the rate at which insoluble biopolymers are decomposed (21). In this regard, soluble intermediates of the anaerobic decomposition process (e.g., sugars, acetate, H_2) are normally found in small amounts, if detected at all, in anaerobic ecosystems such as the rumen (11, 23) or lake sediments (4). Thus, from a kinetic standpoint, interspecies hydrogen transfers that influence the rate at which insoluble polymers are decomposed may be of more environmental consequence than those involving mixed cultures grown on soluble substrates.

The O/R index of the *C. thermocellum* monoculture varied between 0.70 and 0.75. Although the C_2/C_1 ratio suggests that a C_1 compound may have been underestimated in the product balance, it was probably not CO_2, since the same method of CO_2 analysis did not yield an oxidized product deficit in the coculture. Formic acid, although not detected in either culture, may have been formed in trace amounts. However, trace quantities would not be sufficient to correct the low O/R index. This O/R value is within the range reported for pure cultures of several other chemorganotrophic anaerobes, including *Clostridium cellobioparum* (6), *Ruminococcus albus* (12), and the S organism isolated from the "*Methanobacillus omelianskii*" symbiosis (19). Coculturing of *C. thermocellum* with *M. thermoautotrophicum* resulted in an increase in the O/R index to 1.04 to 1.15. An increased O/R index has also been reported in *C. cellobioparum* upon coculture with *Methanobacterium ruminatium* (6).

The altered fermentation patterns in the *C. thermocellum/M. thermoautotrophicum* coculture is in general agreement with the concept of interspecies H_2 transfer (23). The methanogen is responsible for channeling electron flow away from ethanol and toward H_2. However, because *C. thermocellum* is not inhibited by H_2, it does not require the presence of a methanogen for adequate growth. Thus, the growth-dependent interactions reported between H_2-

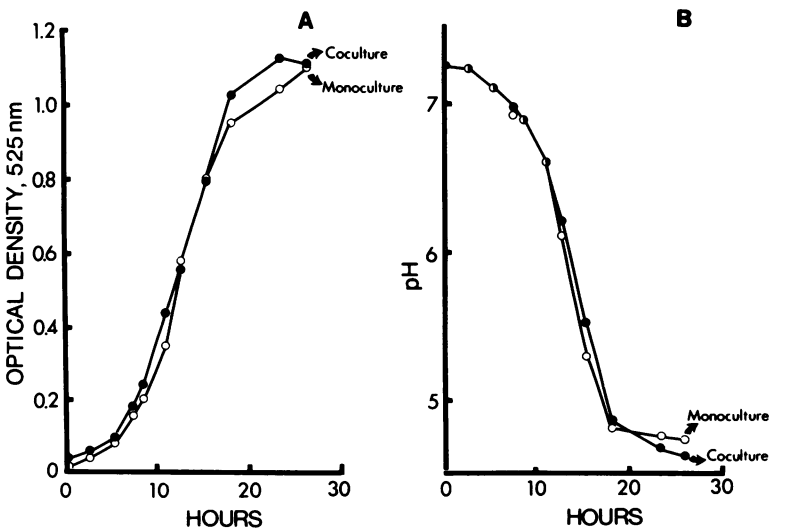

FIG. 4. *Fermentation of cellobiose by C. thermocellum monocultures and C. thermocellum/M. thermoautotrophicum cocultures. Cultures were grown in flasks that contained 100 ml of medium.*

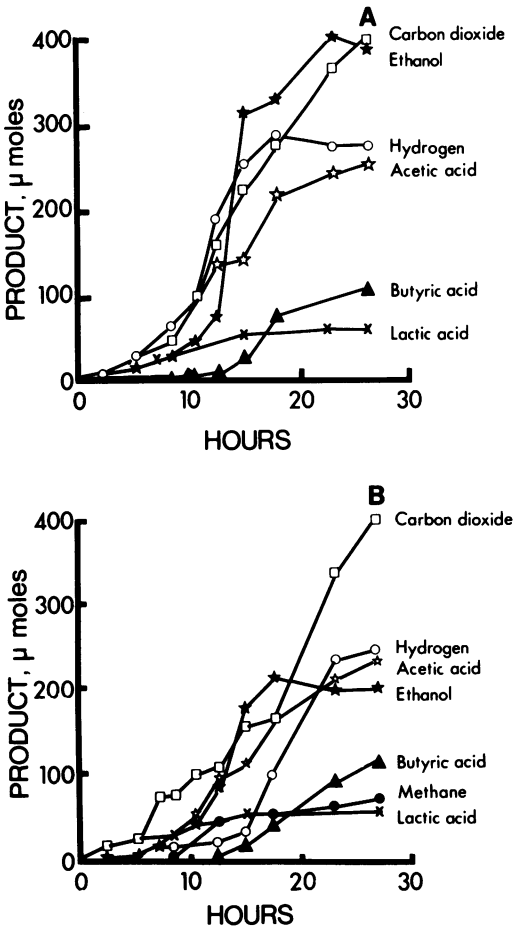

FIG. 5. *Products of cellobiose fermentation by C. thermocellum grown in the absence (A) and presence (B) of M. thermoautotrophicum. Cultures were grown in anaerobic tubes that contained 12 ml of medium. Results are expressed in micromoles of product per tube.*

inhibited chemoorganotrophs and methanogens (6, 19) was not observed in the thermophilic system studied here.

The mechanism of H_2-inhibited growth is somewhat enigmatic. Wolin (23) has suggested that removal of H_2 is required to allow NADH oxidation by the following reaction: $NADH + H^+ \rightarrow NAD^+ + H_2$ $\Delta G^{\circ\prime} = +4.3$ kcal, where NAD^+ is oxidized NAD. When grown on ethanol, the S organism isolated from "*Methanobacillus omelianskii*" relies on this reaction as its sole electron disposal route; thus, it is strongly inhibited by H_2 and growth increases greatly upon coculture with a methanogen (3, 19). However, other H_2-inhibited anaerobes (e.g., *C. cellobioparum*) have other mechanisms of reoxidizing NADH. The H_2 inhibition observed in *C. cellobioparum* may be due to an extreme sensitivity to other reduced fermentation products (e.g., ethanol) which are formed by NADH oxidation when the reaction $NADH + H^+ \rightarrow NAD^+ + H_2$ becomes thermodynamically unfavorable.

It is uncertain whether or not *C. thermocellum* generates H_2 via NADH oxidation. Monocultures produced 0.85 H_2 per glucose-equivalent fermented. However, enough methane is produced in coculture to account for 2.25 H_2 per glucose equivalent fermented, assuming that 4 mol of H_2 are required to produce 1 mol of methane. Theoretically, pyruvate oxidation could account for up to 2 mol of H_2 per mol of glucose, if no pyruvate was removed as lactic acid. This indirect evidence suggests that *C. thermocellum* monocultures produce H_2 exclusively from pyruvate, whereas cocultures produce H_2 by oxidation of pyruvate and possibly NADH.

Chung (6) has shown that the growth rate of *C. cellobioparum* is increased upon coculture with *M. ruminantium*, presumably as a result of greater ATP production associated with the observed increase in acetic acid formation. We have shown that coculturing of *C. thermocellum* with *M. thermoautotrophicum* in cellulose media also causes increased acetic acid production, and that the growth rate of the coculture appears slightly faster than that of the monoculture. Furthermore, the earlier appearance of cellulolytic activity in the coculture may be a consequence of greater ATP production. Utilization of additional ATP by the *Clostridium* in the coculture would result in an increased rate of growth of cellulose production, and of cellulose degradation.

The data presented here demonstrate that mixed cultures of *C. thermocellum* and *M. thermoautotrophicum* are capable of converting cellulose to methane and acetic acid. Preliminary results, not presented here, indicate that the coculture can degrade certain cellulosic waste materials at rates comparable to those for pure cellulose. Although it is difficult to extrapolate these small-scale, batch-culture results to industrial fermentation conditions, the data suggest that this coculture may be useful in a thermophilic bioconversion system. Thermophilic processes offer several advantages over mesophilic processes, including faster rates of substrate turnover and gas production (7, 18) and better control of pathogens and contaminants (7). The coculture described here may be applicable to a bioconversion process if culture conditions, especially pH, were adequately con-

trolled, or if it were used in combination with other thermophilic bacteria, especially methanogenic species that rapidly utilize acetate.

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